THE FLAVORING ETHYL MALTOL, FOUND IN E-CIGARETTES, MEDIATES METAL CELLULAR TOXICITY

by

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Abstract

Background: Currently 3.2% of the US adults (over 18) and 21% of high school students use electronic cigarettes (EC). Use of EC, in high school students, is thought to be driven by their many attractive flavors. One specific flavoring of interest is ethyl maltol, found in approximately 50-80% of the e-liquids. This flavoring has been found to complex with heavy metals and facilitate their uptake into human cells. Recent studies have demonstrated that a wide range of heavy metals, including copper (Cu) are present in both EC vapors and liquids, at potentially harmful levels.

Our goal was to investigate cell death, DNA damage and cellular stress responses in cells exposed to Cu and ethyl maltol.

Methods: Cell death was evaluated in CALU-6 cells with varying concentrations of Cu and 6mM EM using an MTT assay. Apoptosis was assessed using Annexin V and PI staining combined with flow cytometry on CALU-6 cells treated with 5μM Cu. Western blots were used to assess the pathway of apoptosis and DNA damage. Additionally, we investigated ethyl maltol and Cu ability to induce DNA damage and cellular stress responses using immunocytochemistry and western blots.

Results: We successfully demonstrated ethyl maltols ability to induce DNA damage, in CALU-6 cells in the presence of Cu. Ethyl maltol induced cell death in the MTT assay and apoptosis was confirmed as the mechanism of cell death. qPCR revealed a four-fold increase of ferritin light chain and eight-fold increase of heme oxigenase-1 RNA levels. Finally, heme oxigenase-1 protein levels were higher in 5μM Cu with 3mM EM treated CALU-6 cells when compared to the control.

Conclusions: Ethyl maltol in the presence of Cu induces apoptotic cell death at levels relevant to what is found in EC. DNA damage caused by Cu in the presence of EM could mean that exposure can result in carcinogenic effects.

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1.Background

Electronic Cigarettes (EC) are battery powered devices that use a metallic coil to vaporize a liquid (e-liquid), consisting of propylene glycol, vegetable glycerin, and sometimes flavorings and/or nicotine (Farsalinos et al., 2014). Currently, there are a variety of devices and e-liquids to choose from. Devices can be classified into three main types: tanks/mods, rechargeable, and disposable ("About Electronic Cigarettes (E-Cigarettes) | Smoking & Tobacco Use | CDC," n.d.). Within each category there are many different brands to choose from. Additionally, there are several brands and types of juice or e-liquid consisting of different ratios of propylene glycol (PG) and vegetable glycerin (VG), as well as different flavorings and/or nicotine concentrations.

In 2019 over 5 million U.S high and middle school students reported using EC, making the incidence of use higher among teenagers than adults ("About Electronic Cigarettes (E-Cigarettes) | Smoking & Tobacco Use | CDC," n.d.). In 2017 rates of adult reported EC use were 2.8% of the total population (Health, 2019). Of this 2.8%, 58.8% currently smoke traditional combustible cigarettes and EC, 29.8% were formally regular smokers and 11.4% had not previously smoked (Health 2019). Increase in EC use is thought to be driven by several factors: 1) Consumers are switching from traditional cigarettes in hopes of quitting or reducing the amount they smoke (Polosa et al., 2017) 2) Perception that EC are less harmful and less addictive than traditional combustible cigarettes (Farsalinos et al., 2014) 3) Companies market EC as tobacco harm reduction products to persuade individuals into switching, despite limited and conflicting evidence on these claims (Schraufnagel et al., 2014). Of particular interest to us are flavorings found in EC. Through the use of sweet flavorings, EC were highly marketed toward youth (Products, n.d.-b). As a result, there has been a lot of scrutiny surrounding flavorings especially since the recent manifestation of vaping associated illness (EVALI) ("About Electronic Cigarettes (E-Cigarettes) | Smoking & Tobacco Use | CDC," n.d.).

In 2016 the FDA developed policy allowing regulation of EC's under the Family Smoking Prevention and Tobacco Control Act (Products, n.d.-a). This regulation allows the FDA to implement standards and regulation on advertising as well as the composition of EC. Despite the FDA's ability to regulate these products, there is insufficient data for adequate regulation. Notwithstanding this insufficient data, it is important to note that studies have found that EC are not as harm free as they are marketed to be (Canistro et al., 2017; Lee et al., 2018a; Sussan et al., 2015), while others have not seen health effects (Polosa et al., 2017). In order to address this public health problem, more information on the chemical composition and toxicity of these products is needed for correct regulation and accurate risk assessment.

Epidemiologic studies have reported conflicting evidence on the health implication of EC. A three-and-a-half-year cohort study found limited evidence of lung injury, inflammation and respiratory function of EC users who were not previous smokers (Polosa et al., 2017), while another study found that healthy smokers who used an EC for five minutes had increased impedance, peripheral airway flow resistance and oxidative stress (C.I. et al., 2012). Conflicting results of these two studies could be due to the fact that one study looked at EC users with smoking history and the other looked at EC users with-out a smoking history. Health effects in previous smokers is concerning due to the fact that one in six former smokers and one in four current smokers use EC (Schoenborn et al., 2014). Nonetheless these epidemiologic studies are short term studies, while health effects concerning EC are expected to be from to chronic long-term exposure (Health, 2019).

In addition to these epidemiological studies several experimental studies have been performed to determine the cytotoxicity of EC. Canistro and colleagues (2017) found that exposure to EC vapors resulted in an increase in several cytochrome p450 enzymes in the lungs of rats that when extrapolated to humans would result in increased risk of lung cancer (Canistro et al., 2017). Additionally, a reduction in antioxidant enzymes as well as phase II enzymes such

as glutathione S-transferase was also observed in the same study (Canistro et al., 2017). Based on this information authors stated that EC are not as safe as they are marketed to be (Canistro et al., 2017). Other studies have demonstrated evidence of oxidative stress caused by EC in the lungs of mice models with sub-chronic exposure of 1.5 hrs twice daily for two weeks (Sussan et al., 2015).

Several studies have compared the genotoxicity of traditional cigarettes to EC. Thorne and colleagues concluded that EC did not significantly increase double strand breaks in a human lung epithelial model (Thorne et al., 2017). Another research group found that tobacco products, both EC and traditional cigarettes, resulted in an increase of DNA adducts and reduced DNA repair mechanisms in a mouse model and human cells (Lee et al., 2018). Furthermore, scientists have concluded that non-combustible tobacco products have a substantially reduced genotoxic effect in an oral cavity cell line when compared to traditional cigarettes (Gao et al., 2014).

Most recently, a study concluded there is a strong correlation in the number and types of flavorings in EC and cell death (Hua et al., 2019). Many of these added flavoring fall within the "generally recognized as safe (GRAS)" category for oral ingestion. However, chemicals placed in this category are evaluated based on conditions for which they are intended for ("The Safety Assessment and Regulatory Authority to Use Flavors: Focus on E-Cigarettes | FEMA," n.d.). Meaning that these chemicals are classified as safe for ingestion and not for inhalation. Diacetyl is an example of GRAS certified food flavoring that has previously caused health issues when inhaled. Doses at which diacetyl was deemed safe for ingestion were inhaled by factory works and resulted in bronchiolitis obliterans, also known as "popcorn lung"(Barrington-Trimis et al., 2014). This example stresses the need for a better understanding of the inhalation toxicology of constituents found in EC due to the fact different routes of exposures can result in varying toxicological effects.

Ethyl maltol (EM) is a specific food flavoring of interest to us, that has been found to be among the most cytotoxic flavor chemical in EC (Hua et al, 2019). EM is a common food additive that is GRAS certified and has been found in approximately 50%-80% of e-liquids at concentrations around 5mg/mL (Hua et al., 2019; Behar et al., 2018). It has previously been shown that EM is aerosolized in EC aerosols at an efficiency of 39-70% (Omaiye et al., 2019; Behar et al., 2018). Typically, widely used in the food industry, EM is used as an aroma and flavor enhancer (Gralla et al., 1969). Initial toxicity studies found no adverse health effects and concluded it had a wide margin of safety (Gralla et al., 1969). Metabolic studies on dogs concluded that EM is readily metabolized and excreted from the body within 24hr of administration (Rennhard, 1971). Later studies showed that EM complexes with and increases uptake of iron through the intestines (Barrand et al., 1987). More recently a mouse study exploring oral exposure of iron-ethyl maltol complexes, reported lesions of the liver and kidneys (Li et al., 2017). Additionally, EM has also been shown to complex lead and enhance uptake in human erythrocytes (Simons, 1993).

Presence of EM in EC liquids and aerosols is a cause for public health concern due to a recent study demonstrating the presence of metals such as Cu, Fe, Cr and Pb in both EC aerosols and liquids, at potentially harmful levels (Olmedo et al., 2018). Additionally, it was shown that individuals who use EC more frequency had higher concentrations of metals present in their urine when compared to individuals who used EC less (Aherrera et al., 2017). Due to the known properties of EM we suspect that EM in the presence of these metals has the potential to increase metal uptake in cells.

Many metals such as Cu and Fe are essential for regular cellular processes. However, at high concentrations these metals can become toxic and result in a cellular stress response. Iron, in excess, has been shown to increase the production of free radicles and oxidative damage in cells (Britton et al., 2002). Toxicity to large amounts of copper has been described and include GI

and well as liver damage (Atsdr, n.d.). Additionally, Cu has the ability to create reactive oxygen species (ROS) which can result in DNA damage as well as other oxidative damage and stress to the cell (Cervantes-Cervantes et al., 2005).

Additional metals which are not essential for cellular processes, present in EC, are Cr and Pb. Pb toxicity is well characterized and known to cause hematopoietic, renal, reproductive and central nervous system problems (Flora et al,, 2012). Moreover, it is well know that environmental Pb exposure has previously been a worldwide issue, and it has been determined that there is no level of Pb exposure which is safe ("Lead poisoning and health," n.d.). Lastly, chronic inhalation of Cr has been shown to result in lung cancer ("Chromium (Cr) Toxicity: Clinical Assessment - History, Signs and Symptoms | ATSDR - Environmental Medicine & Environmental Health Education - CSEM," n.d.).

The goal of this study was to investigate EM's ability to increase oxidative stress, damage to the cell and cell death in the presence of different heavy metals found in EC. We accomplished this through confirming apoptosis as the mechanism of cells death through Annexin V and PI staining, DAPI staining of nuclear blebbing and JNK activation. We also determined that EM in combination with Cu results in DNA double stranded breaks resulting in the activation of ATM. Lastly, we observed the induction of heme oxygenase-1(HO-1) and ferritin light chain, both downstream of the Nrf2 pathway and heme catabolism.

2. Methods

In order to investigate EM's ability to increase oxidative stress and damage to cells in the presence of different heavy metals found in EC, we conducted a series of experiments. First, we investigated the ability of EM in the presence of Cu and Fe to decrease cell viability through a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay in CALU-6 cells. Based on this information we evaluated the mechanism of cell death, in CALU-6 cells after treatment

with 5μM Cu and 3mM EM. Apoptosis was confirmed as the mechanism of cell death using Annexin V and PI staining, fragmented nucelli staining, and the activation of JNK through a western blot. The ability of Cu and EM to induce DNA double stranded breaks was also evaluated in CALU-6 cells by staining for γH2A.x foci. Lastly, we evaluated the ability of Cu and EM to induce gene in the Nrf2 pathway by RT-PCR and western blots.

2.1 Chemicals and reagents

Antibodies used for immunocytochemistry are the following: 1) Anti-phospho-Histone γH2A.x (Ser139) Antibody, mouse monoclonal, clone JBW301 (EMD Millipore; #05-636) 2) anti-mouse (Alexa-Fluor 488). To stain the nuclei and mount for immunocytochemistry ProLong® Gold Antifade agent with DAPI (4′, 6-diamidino-2-phenylindole) was used (Cell Signaling Technology; #8961). Antibodies used for western blotting are the following: 1) Anti-ATM (D2E2) antibody, rabbit monoclonal (cell signaling, # 2873) 2) Anti-phospho-ATM (Ser1981) antibody, clone 10H11.E12 (Millipore Sigma, #05-740) 3) SAPK/JNK antibody, rabbit monoclonal (cell signaling, #9252) 4) Human/Mouse/Rat Phospho-JNK (T183/Y185) antibody, rabbit monoclonal, clone 1006A (R&D systems, MAB1205) 5) p38 MAP antibody, rabbit monoclonal (Cell signaling, #9212) 6) Phopo-p38 MAPK (Thr180Tyr182), rabbit monoclonal (Cell signaling, #9211) 7) IRDye® 680 Goat anit-Mouse IgG secondary Antibody 8) IRDye® 800CW Goat anti-Rabbit IgG secondary Antibody 9) Anti-β-Actin mouse monoclonal (Sigma-Alrdich). Ethyl maltol was obtained from acron organics and kept in a 6M stock in DMSO at -20°C (4940-11-8). Copper was obtained from Sigma in the form of cupric sulfate penta-hydrate (7758-99-8). For Annexin V and PI, FITC Annexin V was obtained from BioLegend (640906) and PI from Life Technologies (P3566). Binding buffer for Annexin V and PI staining contained 10mM HEPES butter 140mM NaCl and 2.5mM $CaCl_2$.

2.2 Cell culture

CALU-6 epithelial cells were obtained from the laboratory of Dr. Robert Casero. Cells were passaged every three days and split at a ratio of 1 to 4 and grown in MEM media supplemented with 5% fetal bovine serum, 1% non-essential amino acids. In all experimental protocols, culture media was supplemented media with penicillin/streptomycin.

2.3 Viability Assay

CALU-6 cells were plated at a density of 2 x10_4 cells/well in 96 well plates. At 24hr or 48hrs after plating, cells were exposed to 3mM EM and Cu or Fe at varying concentrations. Three wells were reserved and treated with 1% sodium dodecyl sulfate (SDS) to measure background (absorbance of 100% cell death). To assess viability, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added in PBS to each well to achieve a final concentration of 12mM. After a 1hr incubation at 37°C, MTT was replaced with 100µL of DMSO and the plates were shaken at room temperature in the dark for 10 minutes. The absorbance was measured on a microplate reader at 540nm (Spectra Max M5, Molecular Devices LLC, US) using SoftMax Pro® software. Percent viability, normalized to the control, was calculated by using equation 1, where A_{sds} is the average absorbance from the SDS background control, A_e is the average absorbance of the experimental treatment and A_c is the average absorbance of the media control.

Equation 1 $$\frac{(A_{SDS} - A_e)}{A_c} * 100 = \% \, viability$$

Statistical significance was determined by preforming a one-way ANOVA test using Prism (GraphPad, La Jalla, CA, www.graphpad.com).

2.4 Annexin V Propidium iodine (PI) staining

CALU-6 cells were plated at a density of $2x10_5$ cells/well in 6-well plates and exposed to 3mM EM and 1.25-5μM Cu 24hr after plating. Adherent cells were removed with trypsin, spun at 300xg for 5 min, and re-suspended in binding buffer consisting of 10mM HEPES buffer 140mM NaCl and 2.5mM $CaCl_2$. 5μL of fluorochrome (FITC)-labeled annexin V and 5μL of a 100μg/μL solution of PI was added. Samples were incubated at room temperature for 15 min in the dark. Samples were then transferred to ice and 400μL of binding buffer was added. Stained samples were analyzed using flow cytometry (Cytoflex, Beckman-Coulter, US). Statistical significance was determined by preforming a one-way ANOVA test using Prism.

2.5 Imunocytochemistry and DAPI staining

CLAU-6 cells were plated at a density of $12x10_3$ cells on glass cover slips in 24 well plates and allowed to adhere for 48hrs. Cells were treated with 3mM EM and 0.5-5μM Cu for 24hrs, washed three times with PBS then fixed with 4% formaldehyde in PBS for 20 minutes. Cells on coverslips were washed with PBS and permeabilized with 0.2% Trition-X 100 in PBS for 10 minutes followed by three washes of PBS and 20 minutes of blocking with 1% BSA in PBS. Cells on coverslips were incubated over night with a 2μg/mL anti-γH2A.x in 1% BSA at 4°C, washed three times with PBS and incubated with 1:200 dilution of (Alexa-Fluor 488) anti-mouse secondary for 1 hour at room temperature. Cells on coverslips were washed three times with PBS and mounted using ProLong® Gold Antifade agent with 4′,6-diamidino-2-phenylindole (DAPI) and allowed to dry over night at room temperature. Images were taken at 20x and 63x oil immersion using a fluorescence microscope (Apotome System, Zeiss, Germany). γH2A.x foci and apoptotic nuclei were quantified via hand counting. For nuclear fragmentation experiments, cell under the same treatment conditions were fixed with formaldehyde, washed and mounded with DAPI.

2.6 Western Blot

Cells were split 1 to 4 in 10 cm_2 plates and allowed to become confluent for 48 hours. Cells were scraped in the culture media and centrifuged at 300xg for 5 minutes to obtain a pellet. Protein was solubilized in 30uL RIPA lysis buffer (Quality Biological®, US) supplemented with a protease Inhibitor Cocktail Set I (Calbiochem®,US) and phosphatase Inhibitor Cocktail Set II, case/5 (Calbiochem®,US). Cells were sonicated and allowed to sit on ice for 30 minutes and centrifuged at 15,000xg for 10 minutes at 4°C. Protein concentration was measured in the supernatant using the Bradford assay and bovine serum albumin as the standard. 90µg protein was subjected to SDS-PAGE on a 4-12% protein gradient gel and 200 V. Protein was transferred to nitrocellulose membranes at 200 V for 2 hours at 4°C. Membranes were blocked with 1X PBS containing 1% casein (Bio-Rad, US) for 20 minutes at room temperature and incubated with primary antibodies overnight at 4°C. Secondary antibodies were diluted 1:10000 in 1% casein 1X PBS blocking buffer for 1hr at room temperature. Antibody binding was determined by digital fluorescence with the LI-COR Odyssey® (LI-COR Bioscience, US).

2.7 RT-PCR

Cells were plated at $6x10_5$ cells/well in 96 well plats and exposed to 3mM EM and Cu at 24hr after plated. Total RNA was extracted from adherent CALU-6 cells with TRIzol Reagent. Following phase separation using chloroform, the RNA phase was isolated and precipitated with 100% isopropanol. The pellet was washed with 75% ethanol and levels of RNA were quantified with a spectrophotometer (NanoDrop, Thermo-Fisher, US). First-strand cDNA was synthesized using a kit from Thermo Fisher.

Gene expression was quantified and analyzed using CFX Manager software (Bio-Rad, US) to determine the relative quantity (ΔCq) and normalized with housekeeping gene RPLPO to determine expression (ΔΔCq).

The primers sequences for amplification of the target genes can be found in table 1 below.

Table1. Revers and forward primers used for RT-PCR.

RPLPO	Forward: 5'- GCA GCA TCT ACA ACC CTG AAG -3'	Reverse: 5'- CAC TGG CAA CAT TGC GGA C -3'
HO-1	Forward: 5'- AAG ACT GCG TTC CTG CTC AAC -3'	Reverse: 5'- AAA GCC CTA CAG CAA CTG TCG -3'
FTL	Forward: 5'- CAC CTG ACC AAC CTC CAC AG -3'	Reverse: 5'- CGT GCT TGA GAG TGA GCC TT -3'
GCLMA	Forward: 5'- TGT CTT GGA ATG CAC TGT ATC TC -3'	Reverse: 5'- CCC AGT AAG GCT GTA AAT GCT C -3'
NQQ1	Forward 5'- GAA GAG CAC TGA TCG TAC TGG C-3'	Reverse 5' – GGA TAC TGA AAG TTC GCA GGG-3'

3. Results

3.1 Viability Assay

An MTT assay was used to assess the effects of EM and metals on cell viability. A significant decrease in viability was observed in cells treated with Cu at 3μM and EM (Figure 1). No viable cells were remaining for cells treated with Cu at 10μM Cu and EM (Figure 1). Treating cells with Cu alone at 10μM or 3μM did not affect cell viability (Figure 1). Viability was not affected in cells treated with EM and Fe even at concentrations of 25uM (Figure 1).

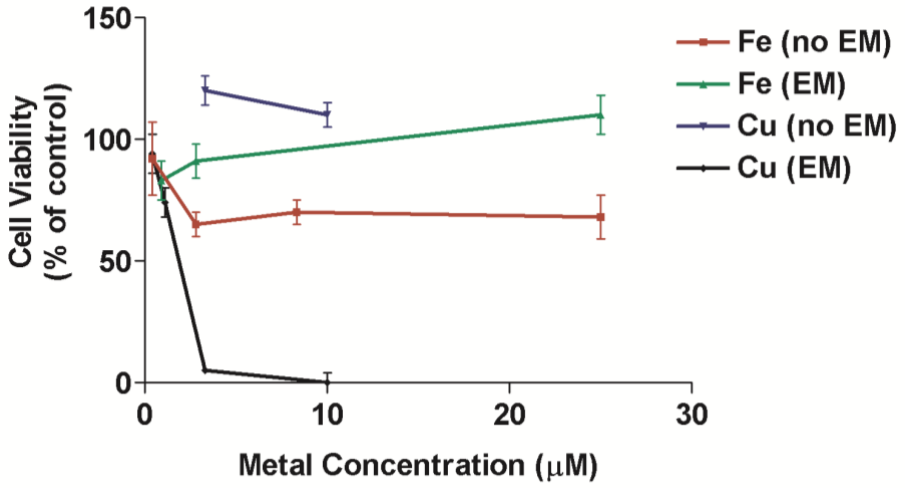

Figure 1. Viability in cells treated with Cu and EM decreased when compared to Cu only treated cells. However, Fe and EM treated cells did have reduced viability when compared to Fe only treated cells. MTT assays were done on CALU-6 cells exposed for 48 hours to Fe or Cu at varying concentrations with and without 3mM EM. Percent viability was calculated by dividing the O.D of treated cells by the O.D of non-treated cells and multiplying by 100.

3.2 Annexin V and PI staining

Apoptosis, one of several mechanisms that causes cells to die, was confirmed as the specific mechanism of cell death using Annexin V and PI staining. A statistically significant increase was observed in cells undergoing early stage apoptosis in cells treated with 5μM Cu and 3mM EM (Figure 2). At lower concentrations of Cu, there appeared to be a trend. This trend summarized in Figure 2 is shown in dot plots in Figure 3. Additionally, a similar trend was observed of late stage apoptotic by being both Annexin V and PI positive (Figure 3). Late stage apoptotic cells are those in the upper right quadrant of each individual plot labeled A-H in Figure 3.

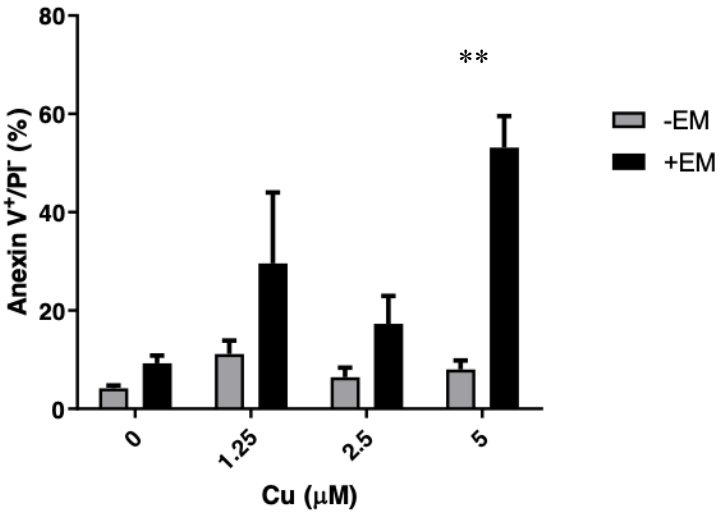

Figure 2. Ethyl maltol in the presence of Cu increases the number of CALU-6 cells undergoing apoptosis. CALU-6 cells were treated for 24 hours with 5 μM Cu and 3mM EM Annexin V and PI staining was analyzed using Flow Cytometry. Data are the mean of three replicates +/- S.E.M ANOVA and the Turkey post-test. (**P<0.01)

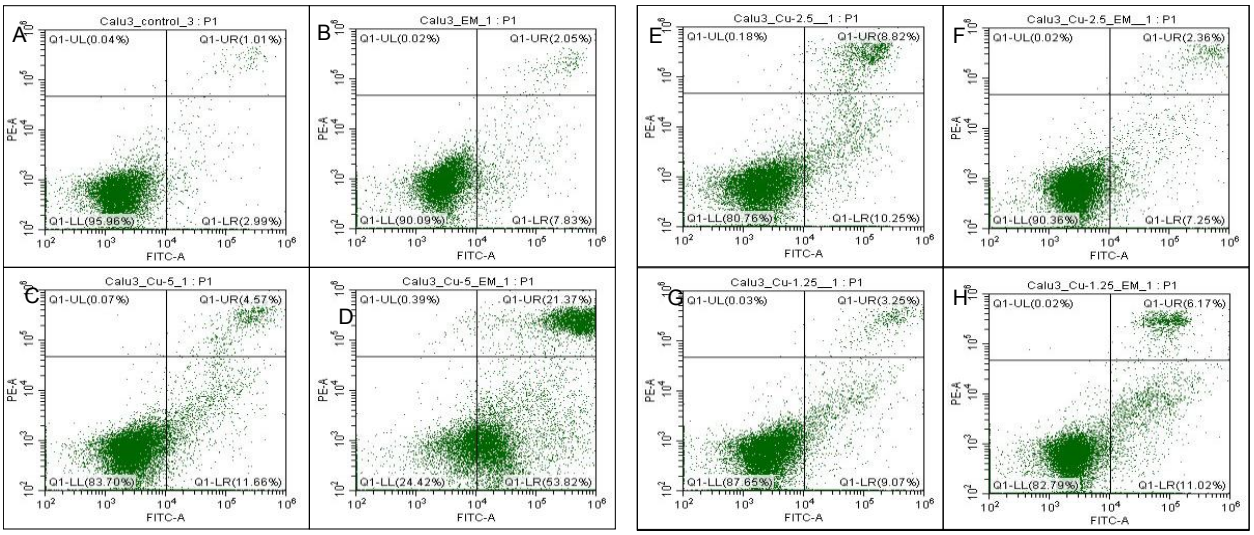

Figure 3. CALU-6 cell treated with indicated Cu concentrations and 3mM ethyl maltol (EM) undergoing apoptosis and necrotic cells. CALU-6 cells were treated for 24 hours with 5μM Cu and 3mM EM Annexin V and PI staining was analyzed using Flow Cytometry. A) Control B) EM only C) 5μM Cu D) 5μM + EM E) 2.5μM Cu F) 2.5μM + EM G) 1.25μM Cu H) 1.25μMc Cu + EM. Cell in the lower right quadrant indicate Annexin V positive cells, upper right indicates Annexin V and PI positive cells, upper left indicates PI positive cells for all plots.

3.3 Imunocytochemistry and DAPI staining

DAPI was used to detect nuclear fragmentation, which indicates a later stage of apoptosis. An increase was observed in the number of cells displaying apoptotic nuclei after treatment with EM and 5μM Cu for 24 or 48hrs (Figure 4 I C and D II C and D). At lower concentrations of Cu, fewer

apoptotic nuclei were observed (Figure 4 I E-F and II E-F). This is consistent with the trend observed in the Annexin V and PI staining shown in Figures 2 and 3.

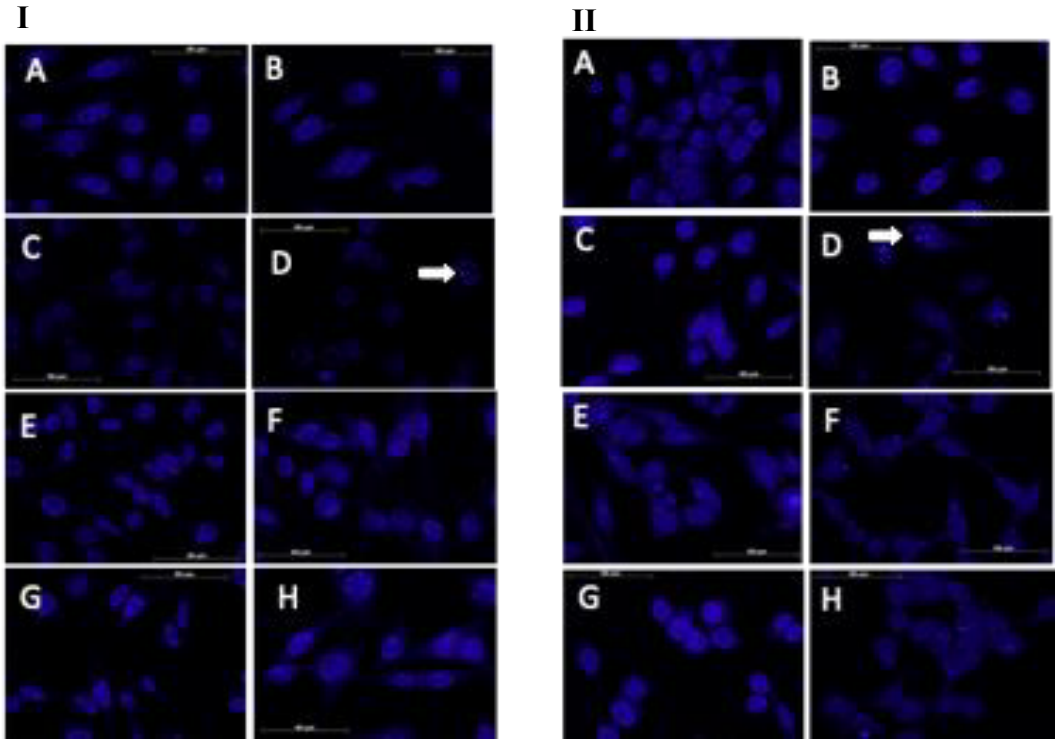

Fig 4. Detection of fragmented nucelli in CALU-6 cells treated with 5 μM Cu and 3mM EM for 24 and 48 hours. Pictures under I indicate cells treated for 24 hours, those under II were treated for 48 hours. For both cases treatments are as follows: A) control B) EM only C) 5 μM Cu D) 5μM + EM E) 2.5 μM Cu F) 2.5μM + EM G) 1.25μM Cu H) 1.25 μM Cu + EM. Detected apoptotic nuclei are indicated by white arrows. Pictures were taken at 63x oil immersion using a fluorescence microscope.

The formation of γH2A.x foci is an early event in the repair of single and double strand deletions. An increase was observed in the percentage of γH2A.x foci positive (10 or greater foci) in cells treated with 0.5- 0.16μM Cu and 3 mM EM when compared to controls (Figure 5 D and F). A smaller increase was observed in the number of cells with foci after treatment with EM alone compared to controls (Figure 5B). Foci were not observed in cells treated with Cu alone (Figure 5 C and E).

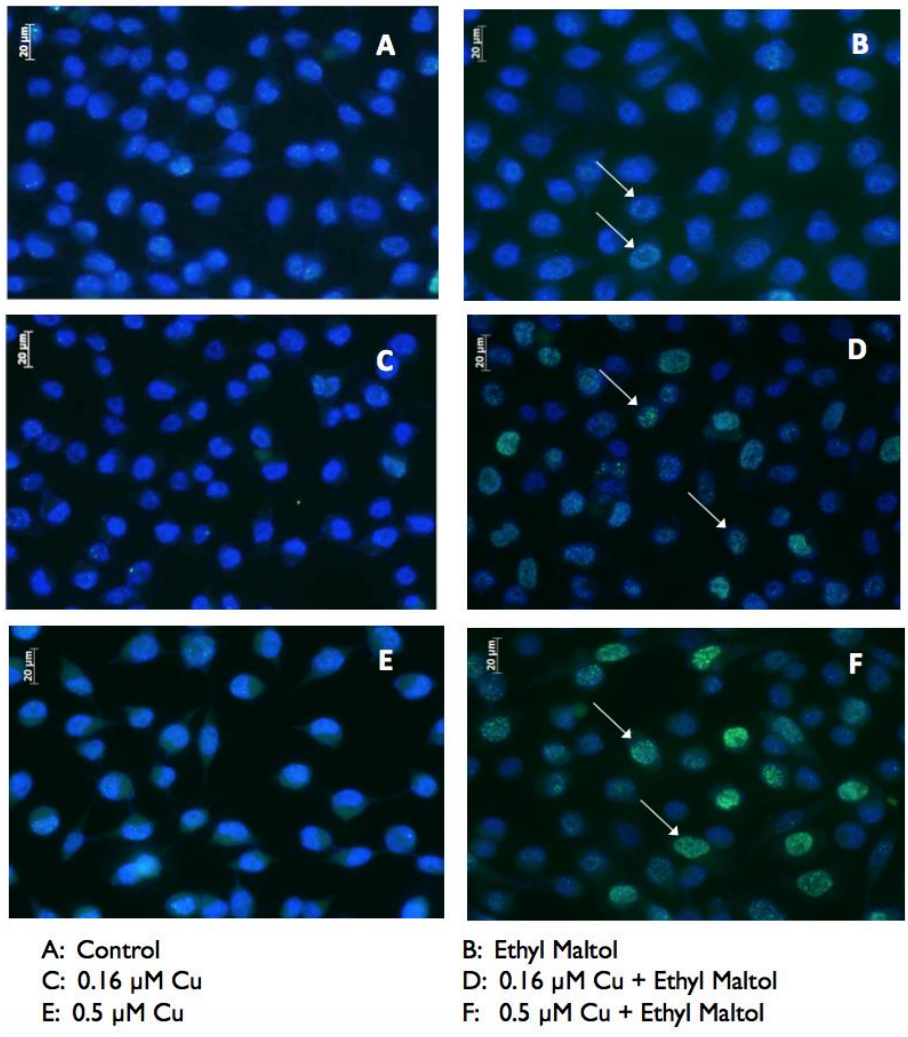

Figure 5. γH2A.X foci (Green) in CALU-6 cells treated with for 24 hours with varying concentration of Cu and 3 mM EM show approximately 40% more foci in cells treated with Cu and 3mM EM. Cell treated with 0.16μM Cu and EM show 44% of cells being γH2A.x positive. Cells treated with 0.5μM Cu and EM show 49% of cells being γH2A.x positive CALU-6 cells were stained with DAPI (Blue) and γH2A.X (Green) histone modification to detect the repair process of double stranded breaks. γH2A.x positive cells (arrows) are quantified as cells with 10 or more foci.

3.4 Activation of protein kinases

We examined the p38 and JNK pathways for their involvement in apoptosis. An increase was observed in pJNK relative to JNK in cells treated with EM and 5uM Cu. An increase was not observed in p38. (Figure 6). The increase in pJNK indicates that EM combined with 5uM Cu activates the JNK pathway

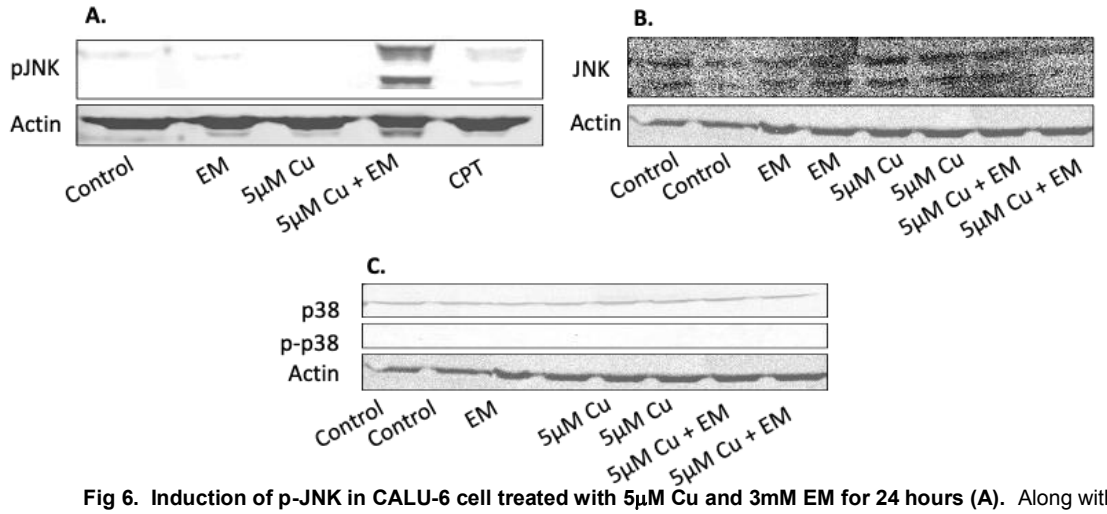

Fig 6. Induction of p-JNK in CALU-6 cell treated with 5μM Cu and 3mM EM for 24 hours (A). Along with the detection of an unchanged amount of JNK in samples treated under the same conditions (B). In both phosphorylated and unphosphorylated forms, the top JNK band indicates p54 JNK and the bottom band p46 JNK. Both antibodies detect total JNK, indicated the activation of JUNK1,2 and 3. Treatment with EM and Cu does not induce phosphorylation of p38 (C).

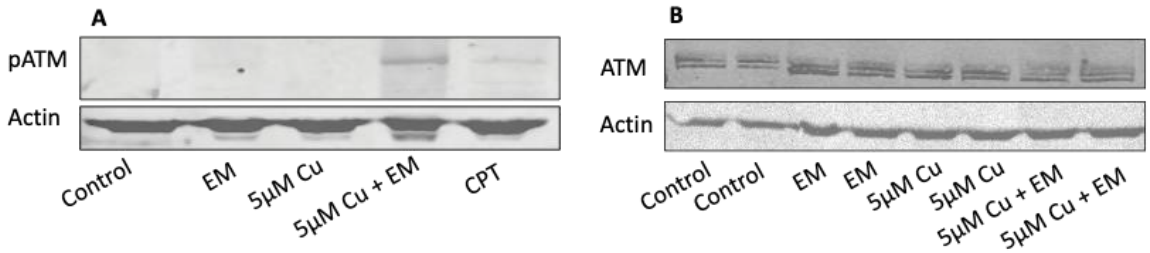

Fig 7. Induction of p-ATM in CALU-6 cell treated with 5μM Cu and 3mM EM for 24 hours (A). Along with the detection of a slight decrease in unphosphorylated ATM under treatment conditions which p-ATM was detected (B).

ATM is a protein kinase that catalyzes the phosphorylation of H2A to γH2A.x. ATM activation involves autophosphorylation and is often in response to DNA damage such as deletions. An increase was observed in phosphorylated ATM without changes in levels of ATM in cells treated with 5μM Cu and 3mM EM for 24 hours (Figure 7).

3.5 Induction of antioxidant defense mechanism

An increase in oxidative stress is a potential mechanism to explain increases in DNA damage and apoptosis. An eight-fold and four-fold increase was observed in the induction of two genes in the antioxidant pathway, HO-1 and ferritin light chain respectively (Figure 8). The increase in HO-1

protein indicates HO-1 enzymatic activity is also elevated (Figure 8). No change is observed in levels of NQQ1 and GCLMa, which are also in the antioxidant defense mechanisms (Figure 8).

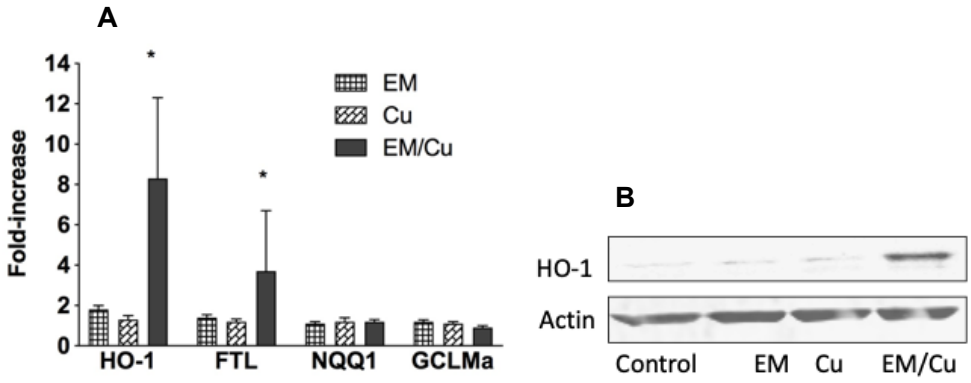

Figure 8. A) Ferritin light chain (FTL) shows a four-fold increase and heme oxygenase-1 (HO-1) shows an eight-fold increase compared to EM only or Cu only treated cells. RT-PCR of CALU-6 cells treated for 24 hours with 5μM Cu and 3 mM EM. Data are the mean of three six replicates +/- S.E.M ANOVA and the Turkey post-test. (*P<0.05, **P<0.01, ***P<001, ****P<0.001) **B) Induction of HO-1 confirmed via western blot in CLAU-6 cells treated with 5μM Cu and 3mM EM for 24 hours.**

4. Discussion

In this study we found that treatment of lung epithelial cells with Cu and EM resulted in decreased cell viability. At the same concentrations, no effect on viability was seen in cells treated with EM or Cu alone. No study, that we are aware of, has examined EM toxicity with Cu. Interestingly, EM in combination with Fe did not affect viability. In contrast, several studies, have reported that influx of Fe, through the creation of ROS, has the ability to induce the JNK pathway and apoptosis (Nakamura et al., 2019; Samet et al., 1998). Additionally, several other studies have reported Fe-mediated toxicity in the presence of EM (Barrand et al., 1987; Li et al., 2017). Currently it is not clear answer why the CALU-6 lung epithelial cell was not responsive to the combination of EM and Fe. Possibly toxicity would have been observed at higher concentrations of Fe. Lung epithelial might be able to tolerate Fe because of the expression of ferritin and other

genes that participate in Fe homeostasis. In contrast, lung epithelial cells possibly do not express sufficient levels of metallothionein, and other genes involved in Cu homeostasis.

Apoptosis is one mechanism to account for the decrease in viability. We have concluded that apoptosis is the specific mechanism of cell death based on the data shown in figures 2, 3, 4 and 6. A hallmark of apoptosis is changes in the plasma membrane, which are thought to allow for recognition for phagocytosis (Martin et al., 1995). Annexin V staining allows for the detection of these membrane changes, specifically, the translocation of phosphatidylserine (PS) from the inside of the membrane to the outside (Martin et al., 1995). Additionally, further supporting the involvement of apoptosis as the mechanism of cells death is the presence of fragmented nuclei, shown in figure 4. Nuclear fragmentation or blebbing is a hallmark of the later stages of apoptosis (Kerr et al.,1972). Lastly, we observed the activation of JNK (Fig. 6), which is a member of the MAP kinase family. JNK is activated by phosphorylation that results in increased expression of genes involved in cell proliferation and apoptosis (Dhanasekaran et al., 2008). Different stimuli have been shown to activate JNK including different stresses, cytokines, growth factors, and other stimuli (Dhanasekaran et al., 2008). Additionally, in figure 6 we did not observe activation of p38, suggesting that cell death as a response to treatment with 5μM Cu and EM is occurring through JNK signaling. Our findings are supported by previous studies. Samet et al., 1998 demonstrated the activation in JNK in bronchial epithelial cells after treatment with Cu. Our study, as well as others, provide evidence to suggest that a range of transition metals, Cr, Fe, Mn, and Ni, have the potential of activating JNK (Samet et al., 1998; Tessier et al.l, 2006). Because transition metals are found in the aerosols generated from EC, JNK activation and apoptosis could be occurring in the lung epithelial of those who use EC.

There are several pathways that induce apoptosis. Our data suggests that apoptosis was due to DNA damage. An increase was observed in the percentage of cells displaying γH2A.x foci (Figure 5). The formation of foci is an early event in the repair of single and double strand

deletions. ATM is one of three kinases that catalyze the phosphorylation of H2A.x to γH2A.x in response to the formation of DNA deletions. The increase in levels of pATM observed in our study indicates ATM was activated (Figure 7). We propose that the combination of EM and Cu activated the DNA damage response through the ATM pathway.

Cu generates reactive oxygen species (ROS) through the Fenton reaction, which would explain DNA deletions (Cervantes-Cervantes et al., 2005). Furthermore, EM and Cu can interact to form hydroxypranone complexes promoting the formation of free radicals, which can also result in oxidative damage and DNA deletions (Bitzer et al., 2017). The combination of EM and Cu could also be evoking other cellular stress responses, such as the activation of genes in the Nrf2 pathway and heme catabolism. We demonstrated the increases in the induction of heme oxygenase one (HO-1) and ferritin light chain (Figure 8). These increases suggest that cells treated with Cu and EM are undergoing oxidative stress. HO-1 has powerful antioxidant capabilities through the catabolism of heme (Choi & Alam, 1996). Interestingly, the NRf2 genes GLCMa and NQQ1 were not induced. Possibly, the induction of Nrf2 genes is time dependent. We did not assess a temporal relation between time of exposure and gene expression. Another possibility is that that the Nrf2 pathway is not involved but rather a different stress pathway.

In summary, our data suggests that cells treated with EM and Cu undergo apoptosis at levels relevant to those found in EC. The induction of apoptosis could be due to DNA damage and other cellular stressors. Cells with DNA damage that fail to undergo apoptosis have the potential of undergoing carcinogenesis.

Public health statement

Previous literature has shown that ECs have the ability to cause DNA damage and reduce DNA repair abilities (Lee et al., 2018). Our data suggests that EC with EM and Cu present have the ability to cause increased DNA damage in users, therefore increase the chance for cells to

undergo carcinogenesis in users. This can increase the incidence of lung diseases such as cancer in EC users, therefore suggesting EC are not as safe as they are marketed to be.

5. Bibliography

About Electronic Cigarettes (E-Cigarettes) | Smoking & Tobacco Use | CDC. (n.d.). Retrieved April 20, 2020, from https://www.cdc.gov/tobacco/basic_information/e-cigarettes/about-e-cigarettes.html

Aherrera, A., Olmedo, P., Grau-Perez, M., Tanda, S., Goessler, W., Jarmul, S., … Navas-Acien, A. (2017). The association of e-cigarette use with exposure to nickel and chromium: A preliminary study of non-invasive biomarkers. *Environmental Research*, *159*, 313–320. https://doi.org/10.1016/j.envres.2017.08.014

Atsdr. (n.d.). *TOXICOLOGICAL PROFILE FOR COPPER*.

Barrand, M. A., Callingham, B. A., & Hider, R. C. (1987). Effects of the pyrones, maltol and ethyl maltol, on iron absorption from the rat small intestine. *Journal of Pharmacy and Pharmacology*, *39*(3), 203–211. https://doi.org/10.1111/j.2042-7158.1987.tb06249.x

Barrington-Trimis, J. L., Samet, J. M., & McConnell, R. (2014, December 17). Flavorings in electronic cigarettes: An unrecognized respiratory health hazard? *JAMA - Journal of the American Medical Association*. American Medical Association. https://doi.org/10.1001/jama.2014.14830

Behar, R. Z., Luo, W., McWhirter, K. J., Pankow, J. F., & Talbot, P. (2018). Analytical and toxicological evaluation of flavor chemicals in electronic cigarette refill fluids. *Scientific Reports*, *8*(1), 8288. https://doi.org/10.1038/s41598-018-25575-6

Bitzer, Z. T., Goel, R., Reilly, S. M., Foulds, J., Muscat, J., Elias, R. J., & Richie, J. P. (2017). Effects of Solvent and Temperature on Free Radical Formation in Electronic Cigarette Aerosols. https://doi.org/10.1021/acs.chemrestox.7b00116

Britton, R. S., Leicester, K. L., & Bacon, B. R. (2002). Iron Toxicity and Chelation Therapy. *International Journal of Hematology*, *76*(3), 219–228. https://doi.org/10.1007/BF02982791

C.I., V., N., A., M., K., V., E., G.N., C., & P.K., B. (2012). Short-term pulmonary effects of using an electronic cigarette: Impact on respiratory flow resistance, impedance, and exhaled

nitric oxide. *Chest*, *141*(6), 1400–1406. https://doi.org/10.1378/CHEST.11-2443

Canistro, D., Vivarelli, F., Cirillo, S., Babot Marquillas, C., Buschini, A., Lazzaretti, M., … Paolini, M. (2017). E-cigarettes induce toxicological effects that can raise the cancer risk. *Scientific Reports*, *7*(1), 2028. https://doi.org/10.1038/s41598-017-02317-8

Cervantes-Cervantes, M. P., Calderón-Salinas, J. V., Albores, A., & Muñoz-Sánchez, J. L. (2005). Copper increases the damage to DNA and proteins caused by reactive oxygen species. *Biological Trace Element Research*, *103*(3), 229–248. https://doi.org/10.1385/BTER:103:3:229

Choi, A. M. K., & Alam, J. (n.d.). *Minireview Heme Oxygenase-1: Function, Regulation, and Implication of a Novel Stress-inducible Protein in Oxidant-induced Lung Injury*. *19% AMERICAN JOURNAL OF RESPIRATORY CELL AND MOLECULAR BIOLOGY* (Vol. 15).

Chromium (Cr) Toxicity: Clinical Assessment - History, Signs and Symptoms | ATSDR - Environmental Medicine & Environmental Health Education - CSEM. (n.d.). Retrieved February 20, 2019, from https://www.atsdr.cdc.gov/csem/csem.asp?csem=10&po=11

Dhanasekaran, D. N., & Reddy, E. P. (2008). JNK signaling in apoptosis. *Oncogene*, *27*, 6245–6251. https://doi.org/10.1038/onc.2008.301

Farsalinos, K., Romagna, G., Tsiapras, D., Kyrzopoulos, S., Voudris, V., Farsalinos, K. E., … Voudris, V. (2014). Characteristics, Perceived Side Effects and Benefits of Electronic Cigarette Use: A Worldwide Survey of More than 19,000 Consumers. *International Journal of Environmental Research and Public Health*, *11*(4), 4356–4373. https://doi.org/10.3390/ijerph110404356

Flora, G., Gupta, D., & Tiwari, A. (2012). Toxicity of lead: A review with recent updates. *Interdisciplinary Toxicology*, *5*(2), 47–58. https://doi.org/10.2478/v10102-012-0009-2

Gao, H., Prasad, G. L., & Zacharias, W. (2014). Combusted but not smokeless tobacco products cause DNA damage in oral cavity cells. *Environmental Toxicology and Pharmacology*, *37*(3), 1079–1089. https://doi.org/10.1016/J.ETAP.2014.03.022

Gralla, E. J., Stebbins, R. B., Coleman, G. L., & Delahunt~, C. S. (1969). *Toxicity Studies with Ethyl Maltol*. Retrieved from https://ac.els-cdn.com/0041008X69900623/1-s2.0-0041008X69900623-main.pdf?_tid=5dac8511-9ad2-4e97-9c96-610da0f0652e&acdnat=1542394708_8e6eddf37bcf47810cfea0fe0b570207

Health, C. O. on S. and. (2019). Smoking and Tobacco Use; Electronic Cigarettes. Retrieved from https://www.cdc.gov/tobacco/basic_information/e-cigarettes/index.htm

Hua, M., Omaiye, E. E., Luo, W., McWhirter, K. J., Pankow, J. F., & Talbot, P. (2019). Identification of Cytotoxic Flavor Chemicals in Top-Selling Electronic Cigarette Refill Fluids. *Scientific Reports*, *9*(1), 2782. https://doi.org/10.1038/s41598-019-38978-w

Kerr, J. F. R., Wyllie, A. H., & Currie, A. R. (1972). Apoptosis: A basic biological phenomenon with wide-ranging implications in tissue kinetics. *British Journal of Cancer*, *26*(4), 239–257. https://doi.org/10.1038/bjc.1972.33

Lead poisoning and health. (n.d.). Retrieved April 10, 2020, from https://www.who.int/news-room/fact-sheets/detail/lead-poisoning-and-health

Lee, H.-W., Park, S.-H., Weng, M.-W., Wang, H.-T., Huang, W. C., Lepor, H., … Tang, M.-S. (2018a). E-cigarette smoke damages DNA and reduces repair activity in mouse lung, heart, and bladder as well as in human lung and bladder cells. *Proceedings of the National Academy of Sciences of the United States of America*, *115*(7), E1560–E1569. https://doi.org/10.1073/pnas.1718185115

Lee, H.-W., Park, S.-H., Weng, M., Wang, H.-T., Huang, W. C., Lepor, H., … Tang, M. (2018b). E-cigarette smoke damages DNA and reduces repair activity in mouse lung, heart, and bladder as well as in human lung and bladder cells. *Proceedings of the National Academy of Sciences*, *115*(7), E1560–E1569. https://doi.org/10.1073/PNAS.1718185115

Li, Z., Lu, J., Wu, C., Pang, Q., Zhu, Z., Nan, R., … Chen, J. (2017). Toxicity Studies of Ethyl Maltol and Iron Complexes in Mice. *BioMed Research International*, *2017*, 1–9. https://doi.org/10.1155/2017/2640619

Martin, S. J., Reutelingsperger, C. P. M., McGahon, A. J., Rader, J. A., Van Schie, R. C. A. A., LaFace, D. M., & Green, D. R. (1995). Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: Inhibition by overexpression of BCL-2 and Abl. *Journal of Experimental Medicine*, *182*(5), 1545–1556. https://doi.org/10.1084/jem.182.5.1545

Nakamura, T., Naguro, I., & Ichijo, H. (2019, September 1). Iron homeostasis and iron-regulated ROS in cell death, senescence and human diseases. *Biochimica et Biophysica Acta - General Subjects*. Elsevier B.V. https://doi.org/10.1016/j.bbagen.2019.06.010

Omaiye, E. E., McWhirter, K. J., Luo, W., Pankow, J. F., & Talbot, P. (2019). High-Nicotine Electronic Cigarette Products: Toxicity of JUUL Fluids and Aerosols Correlates Strongly with Nicotine and Some Flavor Chemical Concentrations. https://doi.org/10.1021/acs.chemrestox.8b00381

Polosa, R., Cibella, F., Caponnetto, P., Maglia, M., Prosperini, U., Russo, C., & Tashkin, D. (2017). Health impact of E-cigarettes: a prospective 3.5-year study of regular daily users who have never smoked. *Scientific Reports*, *7*(1), 13825. https://doi.org/10.1038/s41598-017-14043-2

Products, C. for T. (n.d.-a). Rules, Regulations & Guidance - Family Smoking Prevention and Tobacco Control Act - An Overview. Retrieved from https://www.fda.gov/tobaccoproducts/labeling/rulesregulationsguidance/ucm246129.htm#youth

Products, C. for T. (n.d.-b). Youth & Tobacco - 2018 NYTS Data: A Startling Rise in Youth E-cigarette Use. Retrieved from https://www.fda.gov/TobaccoProducts/PublicHealthEducation/ProtectingKidsfromTobacco/ucm625887.htm

Rennhard, H. H. (1971). Metabolism of ethyl maltol and maltol in the dog. *Journal of Agricultural and Food Chemistry*, *19*(1), 152–154. https://doi.org/10.1021/jf60173a036

Samet, J. M., Graves, L. M., Quay, J., Dailey, L. A., Devlin, R. B., Ghio, A. J., … Reed, W. (1998). Activation of MAPKs in human bronchial epithelial cells exposed to metals. *American Journal of Physiology - Lung Cellular and Molecular Physiology*, *275*(3 19-3). https://doi.org/10.1152/ajplung.1998.275.3.l551

Schoenborn, C. A., & Gindi, R. M. (2014). *Electronic Cigarette Use Among Adults: United States, 2014 Key findings Data from the National Health Interview Survey*. Retrieved from https://www.cdc.gov/nchs/data/databriefs/db217.pdf

Schraufnagel, D. E., Blasi, F., Drummond, M. B., Lam, D. C. L., Latif, E., Rosen, M. J., … Van Zyl-Smit, R. (2014). Electronic Cigarettes. A Position Statement of the Forum of International Respiratory Societies. *American Journal of Respiratory and Critical Care Medicine*, *190*(6), 611–618. https://doi.org/10.1164/rccm.201407-1198PP

Simons, T. J. B. (1993). Lead transport and binding by human erythrocytes in vitro. *Pfl*[illegible]*gers Archiv European Journal of Physiology*, *423*(3–4), 307–313. https://doi.org/10.1007/BF00374410

Sussan, T. E., Gajghate, S., Thimmulappa, R. K., Ma, J., Kim, J.-H., Sudini, K., … Biswal, S. (2015). Exposure to Electronic Cigarettes Impairs Pulmonary Anti-Bacterial and Anti-Viral Defenses in a Mouse Model. *PLOS ONE*, *10*(2), e0116861. https://doi.org/10.1371/journal.pone.0116861

Tessier, D. M., & Pascal, L. E. (2006). Activation of MAP kinases by hexavalent chromium, manganese and nickel in human lung epithelial cells. *Toxicology Letters*, *167*(2), 114–121. https://doi.org/10.1016/j.toxlet.2006.08.015

The Safety Assessment and Regulatory Authority to Use Flavors: Focus on E-Cigarettes | FEMA. (n.d.). Retrieved April 13, 2019, from https://www.femaflavor.org/member-update/safety-assessment-and-regulatory-authority-use-flavors-focus-e-cigarettes

Thorne, D., Larard, S., Baxter, A., Meredith, C., & Gaça, M. (2017). The comparative in vitro assessment of e-cigarette and cigarette smoke aerosols using the γH2AX assay and

applied dose measurements. *Toxicology Letters*, *265*, 170–178.

https://doi.org/10.1016/J.TOXLET.2016.12.006

6. CV

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SUMMARY STATEMENT

Self-motivated, well experienced, graduate student pursuing a Master of Science in Environmental Health Science with excellent written and oral communication skills. A strong interest in laboratory science with a background in chemistry. Applying to Environmental Health PhD programs to further advance my skills and knowledge of Environmental Toxicology.

EDUCATION

Johns Hopkins Bloomberg School of Public Health (JHSPH) *Expected May 2020*
Master of Science in Environmental Health Science
Certificate in Risk Science and Public Policy

State University of New York College of Environmental Science and Forestry (SUNY-ESF) *May 2018*
Bachelor of Science in Chemistry, Focus in Bio-Chemistry

SKILLS AND TECHNIQUES

Computer: R, Microsoft Word. Microsoft Excel, Microsoft PowerPoint, Proficient in Mac and PC operating systems
Microbiology techniques: Sterile technique; Transformation of *E. coli*; Small and medium-scale flask fermentations; Plasmid purifications
Polymer/Organic chemistry techniques: Centrifugation; Soxhlet extractions; Nuclear Magnetic Resonance (NMR); Gel Permeation Chromatography (GPC); High-Performance Liquid Chromatography (HPLC); Dynamic Light Scattering (DLS); Column Chromatography, Inductively Coupled Plasma-Mas Spectroscopy (ICP-MS), Gas Chromatography Mas Spectroscopy (GC-MS)
Biochemistry techniques: Protein Purification; Bradford Assays; Enzymology; SDS-PAGE, Gel electrophoresis, Mammalian cell culturing, PCR, Wester Blot, Dot Blot

WORK EXPERIENCE

Neurotoxicology Lab JHSPH, Baltimore MD *January 2019-Present*
Graduate Researcher
Working to develop an *in vitro* lung model to understand how ethyl maltol mediates metal uptake of metals found in electronic cigarettes
Develop method to quantify semi-volatile organics using GC-MS
Measure and quantify cellular metal uptake using ICP-MS
Preformed western blots to quantify increases and decreases in protein expression

Epigenetic Lab JHSPH, Baltimore MD *August 2018-December 2018*
Graduate Research Assistant
Optimized PCR primers for application of target gene
Preformed gel electrophoresis to confirm amplified PCR products
Extracted and purified DNA bands from gel
Participated in trouble shooting and problem solving
Transfected bacterial cells using vectors made with amplified PCR products, for genome sequencing

Nomura Research Group, Syracuse NY *May 2016-May 2018*
Chemistry Research Assistant
Synthesized, purified, and characterized unnatural fatty acid feedstocks for the bacterial production of polyhydroxyalkanoate (PHA) copolymers
Developed procedure for production of polymer nanoparticles with a diameter of <100 nm for their application as anticancer drugs carriers
Established procedures for the purification and drug-loading of polymer nanoparticles
Calculated, interpreted, and organized drug-loading and size data with the intent of transferring polymer nanoparticle samples to our collaborators
Served as the liaison between our research group and collaborators performing a panel of *in vitro* cell assays

SUNY-ESF Biology Department, Syracuse NY
Teaching Assistant (TA) in Cell Biology *January 2017-May 2017*

Scheduled and ran weekly review sessions for students
Effectively answered student's questions about course material
Completed test and homework grading in a timely manner
Attended classes twice a week to remain up to date on new material

SUNY-ESF Student Affairs, Syracuse NY *March 2016-August 2017*
Orientation Leader
Led small group meetings of +20 first year students in hope of preparing them for their first year at SUNY-ESF
Answered parents and new student questions about welcome events and SUNY-ESF
Assisted first year students with class scheduling and campus navigation queries
Contributed to improvement and new ideas for future SUNY-ESF orientation weeks
Participated in orientation leader training focusing on leadership skills and inclusion

SUNY-ESF Academic Support Services, Syracuse NY *August 2015-May 2017*
Peer Tutor
Instructed students once a week in General Chemistry and Biology, working on understanding new material and test preparation
Scheduled and facilitated weakly tutoring sessions
Shared and demonstrated problem solving skills as well as studying techniques
Participated in tutor training seminars focusing on tutoring practices and leadership skill

SUNY-ESF Dr. Kelley Donaghy's Lab, Syracuse NY August 2015-May 2016
Chemistry Research Assistant
Synthesized bronze clay using wheat flour as binder
Participated in problem solving and data interpretation
Documented detailed steps of experiment in lab notebook
Directed a freshman undergraduate in assisting in on going experiments and data interpretation
Presented work at SUNY-ESF's 2016 Spotlight on Student Research poster symposium

VOLUNTEER EXPERIENCE

Healthy Minds, JHSPH *October 2019- Present*
Elementary School Math Tutor
Tutor third grade math to students in the Baltimore public schools
Play game to practice multiplication, division, subtraction and addition

AXS Honor Society, SUNY-ESF *August 2017-May 2018*
Club Member
Complete a total of fifty hours of community service during each semester as a club member
Submit five tests to SUNY-ESF test files

Volunteer, Syracuse, NY *January 2017-May 2017*
Upstate Medical University
Transferred patients to new and unfamiliar locations within the hospital
Transported young children in a time-efficient and friendly/comforting manner to the children's ER
Assisted ER nurses when patients needed water, blankets and other simple assistance

Chicago 2018 Marathon Finisher *October 2018*
Ran to support the work and mission of the Children's Tumor Foundation
Raise $1,500 through fundraising event for this charity

New York City Marathon Finisher *November 5th, 2017*
Ran for Alex's Lemonade Stand foundation
Raised $3,000 through various fundraiser events for the charity